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ORIGINAL ARTICLE

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Trace metal contamination initiates the apparent auto-aggregation, amyloidosis, and oligomerization of Alzheimer's Aβ peptides

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Abstract Nucleation-dependent protein aggregation (''seeding'') and amyloid fibril-free formation of soluble SDS-resistant oligomers (''oligomerization'') by hydrophobic interaction is an in vitro model thought to propagate β-amyloid (Aβ) deposition, accumulation, and incur neurotoxicity and synaptotoxicity in Alzheimer's disease (AD), and other amyloid-associated neurodegenerative diseases. However, Aβ is a high-affinity metalloprotein that aggregates in the presence of biometals (zinc, copper, and iron), and neocortical Aβ deposition is abolished by genetic ablation of synaptic zinc in transgenic mice. We now present in vitro evidence that trace (≤ 0.8 μM) levels of zinc, copper, and iron, present as common contaminants of laboratory buffers and culture media, are the actual initiators of the classic Aβ1–42-mediated seeding process and Aβ oligomerization. Replicating the experimental conditions of earlier workers, we found that the in vitro precipitation and amyloidosis of Aβ1–40 (20 μM) initiated by Aβ1–42 (2 μM) were abolished by chelation of trace metal contaminants. Further, metal chelation attenuated formation of soluble Aβ oligomers from a cell-free culture medium. These data suggest that protein self-assembly and oligomerization are not spontaneous in this system as previously thought, and that there may be an obligatory role for metal ions in initiating Aβ amyloidosis and oligomerization.

Keywords Aβ amyloid · Aβ oligomerization · Alzheimer's disease · Metal chelator · Trace metal contaminants

Abbreviations Aβ: β-amyloid · AD: Alzheimer's disease · CR: Congo Red · DMEM: Dulbecco's modified Eagle's medium · DTPA: diethylenetriaminepentaacetic acid · ICP-MS: ion coupled plasma-mass spectroscopy · SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis · Th-T: thioflavin-T

Introduction

Alzheimer's disease (AD) is characterized by the abundant deposition of β-amyloid (Aβ) within the neocortical parenchyma and the cerebrovasculature [1]. The Aβ peptides are a group of soluble proteins of considerable amino and carboxyl terminal heterogeneity, found in all biological fluids, with Aβ1–40 being the major species [2]. The neurochemical mechanisms that lead to the age-dependent precipitation of Aβ amyloid in AD brains are still uncertain, but the length of the hydrophobic carboxyl terminus of Aβ is considered to be a key factor in this event. This is because Aβ1–42, which is a minor biological species, is enriched in amyloid [3], and familial AD-linked mutations of amyloid precursor protein (APP), presenilin-1 (PS1), and presenilin-2 (PS2) increase Aβ1–42 production [4, 5]. Therefore, the mechanism of Aβ1–42-mediated amyloid formation is of relevance in understanding AD pathogenesis.

It has been assumed that Aβ1-42 forms amyloid through a simple mechanism of self-association driven by hydrophobic regions of the peptide [6, 7]. This

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mechanism was posited to explain why Aβ1–42 appears more liable to auto-aggregate than shorter Aβ species [8, 9]. Formation of nuclei due to the hydrophobic self-aggregation of Aβ1–42 was also suggested to cause the destabilization of Aβ1–40 in solution [10]. Aβ1–40, which is kinetically stable at 20 μM in solution for 9 d, is destabilized over that interval by seeding with 2 μM Aβ1–42 fibrils [11]. Nucleation-dependent seeding of amyloid formation has been proposed to be applicable to other protein deposition-related disorders such as prion diseases [10, 12].

However, the models that theorize that Aβ precipitation is caused simply through self-association cannot fully explain the age-dependent Alzheimer's Aβ amyloidogenesis. The mere presence of Aβ1–42 is unlikely to be the sole initiator of in vivo amyloid deposition, since the peptide is a normal component of cerebrospinal fluid (CSF). Furthermore, there is no clear evidence of a sustained elevation of Aβ1–42 levels in non-familial forms of AD. Even in familial AD cases where Aβ1–42 levels are elevated, it is difficult to explain why the amyloid deposits are focal (related to synapses and the cerebrovascular lamina media) and not uniform in their distribution. For these reasons, we have suspected that other neurochemical factors, present in the microanatomical environment where Aβ accumulates and dysregulated as a stochastic consequence of aging, must impact upon the Aβ accumulation process in AD.

An alternative explanation for the precipitation of Aβ is its interaction with the brain's dysregulated endowment of biometals. We, and others, have characterized Aβ as a metalloprotein that is precipitated in vitro by submicromolar concentrations of ionic zinc, copper, and iron [13, 14, 15, 16, 17]. Zinc, copper, and iron are markedly enriched (Cu$\approx$0.4 mM, Zn and Fe$\approx$1 mM) in amyloid plaques [18, 19, 20]. Indeed, genetic ablation of neocortical synaptic zinc release dramatically inhibits β-amyloid deposition in Tg2576 mice [21, 22]. Meanwhile, the rat/mouse homologue of Aβ, containing three amino acid substitutions within the metal-binding domain of the peptide, is not precipitated by low micromolar concentrations of either Zn(II) or Cu(II) [14, 17], which may explain why these animals do not develop amyloid deposition with age, even when endogenous mouse Aβ1–42 is over-expressed in the brains of FAD-linked mutant presenilin transgenics [23].

A modified amyloid cascade model attributes the toxicity of Aβ1–42 to the formation of small diffusible Aβ oligomers, which, while soluble, are SDS-resistant, and are also believed to be formed by self-association of Aβ1–42 through hydrophobic forces [24]. Indeed, growing experimental evidence indicates that there are multimeric forms of Aβ extracted from AD brain [25, 26, 27, 28], present in AD CSF [2, 29], and secreted in media [30, 31]. Furthermore, cells expressing mutant presenilin genes generate increased levels of dimeric Aβ [32]. Small, soluble Aβ oligomers, which are both neurotoxic and synaptotoxic [24, 33], accumulate strikingly in AD brain [34]. A recent study shows that Aβ oligomers and other soluble oligomers display a common conformation-dependent and sequence-independent structure that may offer a unified cellular toxicity mechanism [35]. Our recent results demonstrate that redox-active Cu mediates the formation of dityrosine-crossed-linked, SDS-resistant Aβ oligomers [36]. Therefore, metal ions rather than intermolecular forces may also drive the Aβ oligomerization process.

Since Zn(II), Cu(II), and Fe(III) are atmospheric pollutants [37] and surface contaminants that readily contaminate experimental buffers to submicromolar concentrations, we considered the impact that these trace-level Aβ aggregants may have upon the seeding reaction of Jarrett et al. [11]. Herein, we reproduce the original findings of Jarrett et al. [11] and report that, as expected, the addition of Zn(II), Cu(II), and Fe(III) markedly enhances Aβ1–42-initiated seeding of Aβ1–40. However, the presence of diethylenetriaminepentaacetic acid (DTPA), a strong Cu(II), Zn(II), and Fe(III) chelator, abolishes the seeding reaction in the absence of any exogenous metal ions. Further, DTPA attenuated formation of soluble Aβ oligomers in a cell-free culture medium. These findings demonstrate how sensitive the Aβ seeding reaction and oligomerization is to the presence of trace levels of metal ions and suggest an essential role for these trace metal ions in the classic Aβ nucleation reaction and even oligomerization.

Materials and methods

Reagents and preparation

Human Aβ1–40 and 42 amyloid peptides were synthesized by the W.M. Keck Foundation Biotechnology Resource Laboratory (Yale University, New Haven, Conn., USA). The synthetic Aβ peptides were characterized and prepared as previously described [16]. Synthetic Aβ peptides were dissolved $\sim$300 μM in Milli-Q water (Millipore Corporation, Milford, Mass., USA) within 3 h of commencing the experiment, sonicated for 3 min through a water bath to avoid frothing, centrifuged (10 min$\times$10,000 *g*), and the supernatants were collected for determination of peptide concentration as previously described [16]. Sonication and centrifugation are considered to be critical procedures to remove any trace of peptide microparticulate matter.

Metal ion stock solutions [Fe(III)-citrate, Cu(II)-glycine, and Zn(II)-histidine] were prepared by mixing National Institute of Standards and Technology (NIST) standards with ligands (citrate, glycine, and histidine) at a metal/ligand molar ratio of 1:6 in PBS (Sigma; composition: 1.19 mM $CaCl_2$, 0.6 mM $MgCl_2$, 2.7 mM KCl, 1.4 mM KH_2PO_4, 137 mM NaCl, 7.68 mM Na_2HPO_4, "PBS"). The background iron, copper, and zinc concentrations in the PBS were measured by ion coupled plasma-mass spectroscopy (ICP-MS). All metal ion stock solutions were adjusted to pH 7.40 $\pm$ 0.05 before use.

Turbidometric, Congo Red, and thioflavine-T binding assays for Aβ1–42-mediated seeding

To evaluate the effects of metal ions and metal chelators on the Aβ seeding process, either Aβ1–40 (22 μM) or Aβ1–40 (20 μM) with Aβ1–42 (2 μM) were co-incubated in PBS, pH 7.4, in the presence or absence of metal ions (10 μM) and/or the potent metal chelator DTPA (25 μM, Sigma). Log K values for DTPA are Zn(II) 18.2, Cu(II) 21.2, Fe(III) 27.7, Co(II) 18.8, Ni(II) 20.1 (NIST Standard Reference Database 46, version 8.0).

The incubations were performed at 37 °C in a flat-bottom 96-well microtiter plate (Corning Costar Corporation, Mass., USA), and turbidity absorbances (400 nm) were measured daily over a 9-d period using a SpectraMAX Plus microplate reader directed by Softmax PRO version 2.1.0 software (Molecular Devices Corporation, Calif., USA). An automatic 30-s plate agitation mode was selected for the plate reader to evenly suspend the aggregates in the wells before all readings. Precautions for preventing evaporative loss of the solutions were taken.

Congo Red (CR, Sigma) binding was also used to appraise peptide aggregation states during the Aβ seeding process, using an established protocol [38]. The same Aβ mixtures with or without metal ions or DTPA as in the turbidometric studies were prepared under identical experimental conditions (in 1-mL Eppendorf tubes) and CR stock solution (25 μM in PBS) was added to the peptide mixture. Measurement of CR binding was taken immediately, and again after incubation for 10 d. The concentration of CR bound to Aβ aggregates was calculated as follows [39]:

$$C_b(\mathrm{M}) = (A_{540}/25,295) - (A_{477}/46,306) \quad (1)$$

where A_{540} and A_{477} are absorbances at 540 nm and 477 nm, respectively.

Thioflavin-T (Th-T, Sigma) assays of Aβ amyloidosis were performed according to published procedures [38]. Peptide or buffer blank samples (3 μL) that had been either immediately prepared or had been incubated for 10 d, with or without metal ions or DTPA (as above), were added to Th-T (3 μM×297 μL) in PBS. Fluorescence (E_x = 450 nm; E_m = 482 nm) was read immediately by a SPECTRAmax GEMINI microplate spectrofluorometer (Molecular Devices Corporation, Calif., USA).

Western blotting for chelation effects upon metal-mediated Aβ oligomerization

To assess the effects of metal chelation on the formation of Aβ oligomer, either Aβ1–40 (10 μM) or Aβ1–40 (9 μM) with Aβ1–42 (1 μM) were co-incubated in cell-free culture medium [Dulbecco's modified Eagle's medium (DMEM, Nutrient Mixture F-12 Ham, Sigma)], in the presence or absence of DTPA (25 μM, Sigma). The background iron, copper, and zinc concentrations in the medium were measured by ICP-MS. The incubations were performed at 37 °C in Eppendorf tubes for either 0 or 24 h, and the final solutions were centrifuged (15 min×10,000 g) and the supernatant was assayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using WO2 as the primary antibody. The final images were captured and analyzed by a VersaDoc Digital Imaging System (Bio-Rad). To quantify Aβ oligomerization, the band density in each lane was determined by reflectance analysis of the images using the Quantity One imaging analysis software (basic version 4.5.1, Bio-Rad). As a representative oligomeric Aβ species, the dimer band in each lane was selected and its band density percentage in total was calculated and presented.

Results

Aβ1–42-mediated Aβ1–40 seeding with or without the presence of metal ions (iron, copper, and zinc) was first assessed by turbidometric assay (Fig. 1A). In the absence of added metal ions ("Aβ40/42 alone", 2 μM Aβ1–42 in 20 μM Aβ1–40), we observed a clear increase in turbidity on the 4th day of the incubation and the turbidometric change (ΔA_{400}) plateaued at

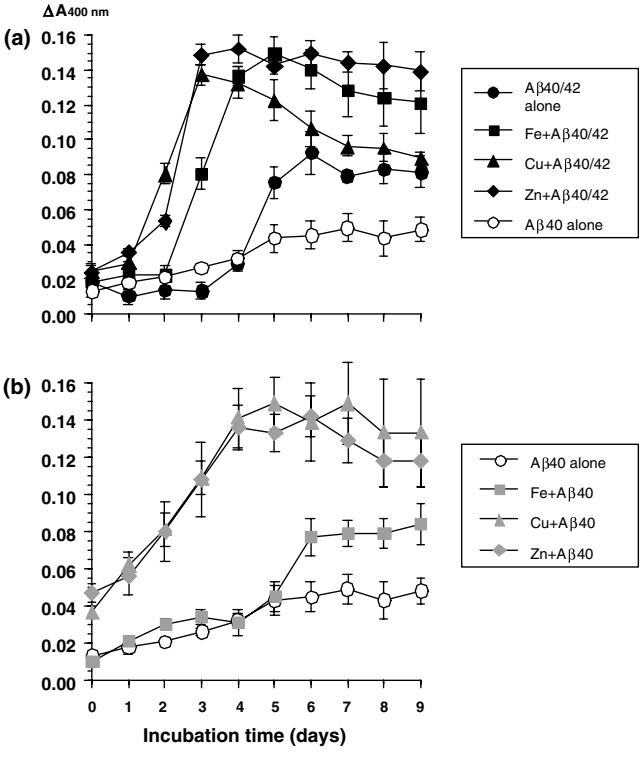

Fig. 1A, B Turbidometric analysis of Aβ seeding: effects of metal ions. Aβ mixtures, (**A**) 20 μM of Aβ1–40 and 2 μM of Aβ1–42 ("Aβ40/42") or (**B**) 22 μM Aβ1–40 ("Aβ40"), were co-incubated as indicated with metal ion complexes [25 μM, Fe(III)-citrate, Cu(II)-glycine, or Zn(II)-histidine], in PBS, pH 7.4, at 37 °C over a 9-d period. The data indicate the mean (±SD, n = 3) turbidity (absorbance at 400 nm) changes against the incubation buffer blank alone

~0.08 absorbance units after 6 d. In contrast, an equimolar solution of Aβ1–40 (Fig. 1A, Aβ40 alone) developed only a small (~0.03 absorbance units) increase in turbidity over the same time frame. These data are in close agreement with the original observations of Jarrett et al. [11].

When the Aβ1–42-mediated Aβ1–40 seeding reactions were performed in the presence of Zn(II), Cu(II), or Fe(III) [10 μM, presented as Zn(II)-histidine, Cu(II)-glycine, and Fe(III)-citrate complexes so as to more closely resemble a biological fluid], the rate of each reaction was markedly accelerated, with peak turbidity achieved on the 4th day in the presence of Zn(II) and Cu(II), and on the 5th day for Fe(III). The peak turbidity (ΔA_{400}) achieved in the presence of any of the three supplemented metal ions tested was approximately double (0.15 units) the peak turbidity in their absence (~0.08 units) (Fig. 1A).

These results are not surprising, since the metal ions at this concentration, under these conditions, would each be expected to induce the immediate precipitation of a proportion of the Aβ in the solution, based upon our previous findings [13, 14, 16, 17, 40]. However, these findings indicate that the kinetics of the Aβ1–42-induced destabilization of Aβ1–40 in solution is accelerated in this environment, perhaps by the increase of nucleated particles that occur when some of the peptide in solution is rapidly induced to aggregate by the biometals.

To test this possibility, a solution of Aβ1–40 alone (22 μM) was treated with the same concentrations of biometals (Fig. 1B), and reacted similarly. Peak turbidity in the presence of Zn(II) or Cu(II) was reached after 4 d and the increase in ΔA_{400} was also ~0.14 units. Fe(III)-induced turbidity was slower, peaking on the 6th day, and exhibited a smaller increase in turbidity ($\Delta A_{400\text{nm}} \approx 0.08$ units). These findings support the likelihood that microparticulate forms of Aβ1–40 alone, in this case induced by biometals, can also initiate the destabilization of a supersaturated Aβ1–40 solution. This metal-mediated reaction is a seeding-type event because it took several days to reach completion, in contrast to the ionic assembly of metal-mediated Aβ aggregates, which reaches completion instantaneously [16, 20]. Fe(III) induces less aggregation of Aβ than Zn(II) or Cu(II) [16, 20], which may explain why the seeding of Aβ is slower in the presence of Fe(III) than in the presence of Cu(II) or Zn(II). Jarrett et al. [11] suggested that the formation of Aβ1–42 nuclei from solutions occurred faster than the formation of Aβ1–40 nuclei, and therefore Aβ1–42 destabilized supersaturated solutions of Aβ1–40. Our data extend that model by showing that nuclei of Aβ1–40 induced by metal ions may substitute for Aβ1–42 in this form of seeding reaction.

Since Zn(II), Cu(II), and Fe(III) markedly accelerated the Aβ seeding reaction, we hypothesized that the destabilization of Aβ1–40 in the absence of added metal ions may be influenced by trace concentrations of these metal ions. Assay of these metals in our experimental buffers by ICP-MS revealed that contamination in the buffer vehicle, even after de-metallation with Chelex resin (Bio-Rad), was 0.18 μM for Fe, 0.076 μM for Cu, and 0.047 μM for Zn. Sources of contamination include vessel surfaces and the atmosphere, and it is very difficult to avoid such trace levels of contamination even with precautions under ordinary laboratory conditions. Also, we have found that synthetic Aβ preparations are usually contaminated with metals, regardless of the source of the synthesis. Typical levels of contamination are (in 10^{-3} moles/mole peptide): for Fe, 17 for Aβ1–40, 90 for Aβ1–42; for Cu, 5.3 for Aβ1–40, 3.6 for Aβ1–42; for Zn, 1.3 for Aβ1–40, 2.5 for Aβ1–42. The metal content of 20 μM Aβ1–40 with 2 μM Aβ1–42 in PBS, pH 7.4, would therefore be expected to be Fe ~0.8 μM, Cu ~0.2 μM, and Zn ~0.1 μM, and other metal ions that aggregate Aβ1–40 could also be present as contaminants in the peptide solution (e.g. Co and Ni) [17].

To test whether trace levels of metal ions contribute to the Aβ seeding reaction, Aβ1–40 (20 μM) with Aβ1–42 (2 μM) was co-incubated in PBS, pH 7.4, in the presence of a potent metal chelator, DTPA (25 μM). DTPA, which has a high affinity for Fe(III), Cu(II), Zn(II), Co(II), and Ni(II) (see Materials and methods), abolished the Aβ1–42-mediated seeding of Aβ1–40 (Fig. 2A). This suggests that the presence of metal ions is obligatory for nucleation to occur. When co-incubated with both DTPA and equimolar concentrations of the metal ions together, the seeding reaction was not inhibited (data not shown), indicating that DTPA exerted its effects by complexing metal ions and not by

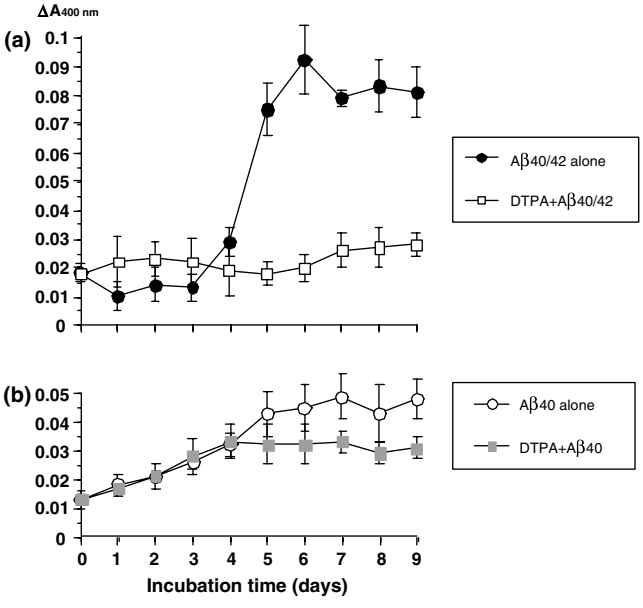

Fig. 2A, B Effects of DTPA on Aβ seeding. Aβ mixtures, (**A**) 20 μM of Aβ1–40 and 2 μM of Aβ1–42 ("Aβ40/42") or (**B**) 22 μM Aβ1–40 ("Aβ40"), were co-incubated as indicated with DTPA (25 μM) in PBS, pH 7.4, at 37 °C over a 9-d period. The data indicate the mean (±SD, $n=3$) turbidity (absorbance at 400 nm) changes against the incubation buffer blank alone

binding to Aβ. When DTPA was co-incubated with Aβ1–40 alone (22 μM), it inhibited the modest turbidity increase that developed in the absence of Aβ1–42 over the 9-d incubation (Fig. 2B). The inhibitory effect of DTPA upon aggregation of Aβ peptides is unlikely due to direct interaction between DTPA and Aβ peptides, as NMR spectra of Aβ1-42 peptide show no change either in the presence or absence of DTPA (data not shown).

To further quantify the effects of these metal ions and DTPA upon the nucleated aggregation of Aβ, we measured the increase in binding of Congo Red to Aβ aggregates formed after incubation for 10 d. There was a marked increase in CR binding to the Aβ1–42-seeded Aβ1–40 mixture (peptide alone, Fig. 3A) that was further increased by co-incubation with Cu(II) or Zn(II), in agreement with the turbidity results (Fig. 1A). Co-incubation with Fe(III) did not significantly increase CR binding above that resulting from Aβ1–40/1–42 incubation alone (Fig. 3A), despite markedly increasing the rate of turbidometric change (Fig. 1A). This suggests that CR binding as a marker of Aβ aggregation may not reflect the same physical phenomena as increased peptide turbidity. Nevertheless, DTPA significantly (*t*-test, $P < 0.001$) inhibited the increase in CR binding to the Aβ1–42-seeded Aβ1–40 mixture, in agreement with the turbidometric data (Fig. 1B), confirming that trace levels of metal ions played an obligatory role in the nucleation of Aβ.

As a specific indicator of β-sheet content of protein amyloid [41], Th-T binding to aggregates formed by the Aβ1–42 seeding of Aβ1–40 following 10-d incubations was measured (Fig. 3B). These data are in agreement with the trends observed in the CR and turbidometric assays performed upon these reactions (Figs. 1 and 2), in that amyloidosis was increased in the Aβ1–40/1–42 mixtures after incubation for 10 d, as expected, and Cu(II) and Zn(II) significantly increased the amount of amyloid detected, whereas only a marginal increase in amyloidosis was induced by Fe(III). In contrast, DTPA inhibited amyloid formation during the incubation period.

We have recently reported that redox-active Cu mediates the formation of dityrosine-crossed-linked, SDS-resistant Aβ oligomers [36]. Similar soluble Aβ oligomers are generated either in cell-free culture medium or conditioned medium from cell cultures [24, 33]. Metal salts are essential ingredients in the cell culture medium. Fe, Cu, and Zn concentrations in a typical DMEM were determined to be 34 ± 7 μM, 0.94 ± 0.13 μM, and 58 ± 11 μM by ICP-MS, respectively. To appraise the role of these metals in the oligomerization of Aβ in culture medium, Aβ1–40 (10 μM) or Aβ1–40 (9 μM) with Aβ1–42 (1 μM) were co-incubated in the cell-free DMEM, in the presence or absence of DTPA (25 μM). Soluble Aβ oligomers were determined by SDS-PAGE using WO2 as a primary antibody. As shown in Fig. 4, Aβ1–40 or Aβ1–40/42 peptides commenced as apparent monomers or dimers in the medium, but apparent oligomerization occurred after 24 h incubation. Oligomerization was exaggerated

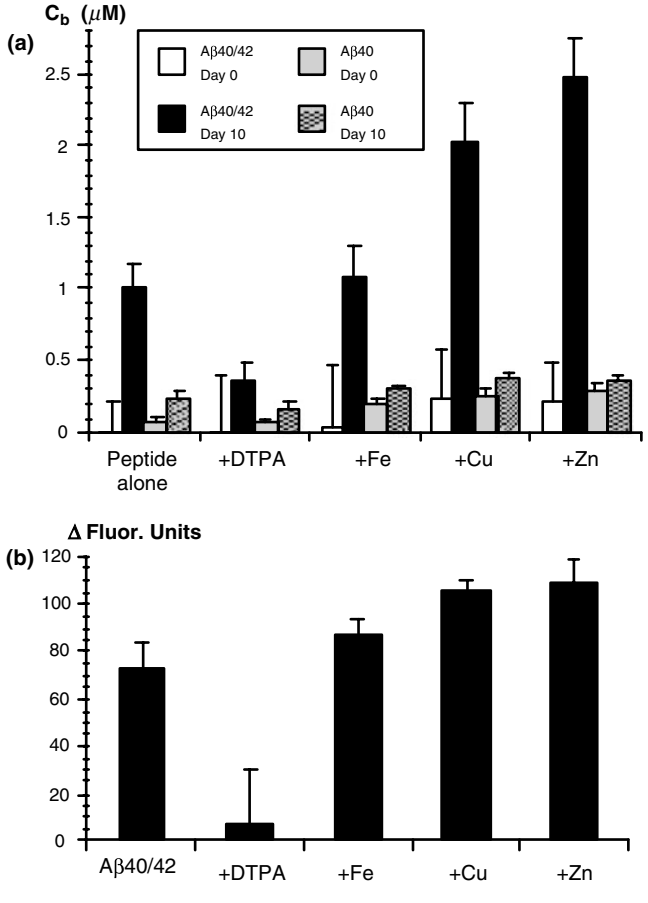

Fig. 3A, B Aggregation and amyloidosis assays of Aβ seeding: effects of metal ions and DTPA. Aβ40/42 (20 μM of Aβ1–40 and 2 μM of Aβ1–42) or Aβ40 (22 μM Aβ1–40) were co-incubated as indicated with metal ion complexes [25 μM, Fe(III)-citrate, Cu(II)-glycine, or Zn(II)-histidine] or DTPA (25 μM), as indicated, in PBS, pH 7.4, at 37 °C for 10 d. Increases in Aβ (**A**) aggregation (measured by CR binding) and (**B**) amyloidosis (measured by Th-T binding and presented as ΔFluor Units after buffer background deduction), were determined. The data indicate the mean ($\pm$SD, $n = 3$) changes against the incubation buffer blank alone

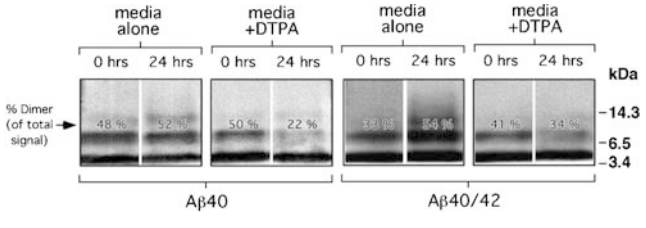

Fig. 4 Western blot analysis of metal-dependent Aβ oligomerization. Aβ40 (10 μM) or Aβ40/42 (9 μM of Aβ1–40 and 1 μM of Aβ1–42) were co-incubated as indicated with or without DTPA (25 μM) in cell-free culture media (DMEM). The incubation was performed at 37 °C for either 0 or 24 h; the solutions were centrifuged (15 min×10,000 *g*) and the supernatant was assayed by SDS-PAGE using WO2 as a primary antibody. The band density in each lane was determined by reflectance analysis of the captured images, and the percentage dimer band density in total was calculated and presented in each lane

in mixtures of Aβ1–40/42, possibly because Aβ1–42 has greater metal-dependent redox activity [42, 43]. Supporting the interpretation that oligomerization was due to metal-mediated chemistry, DTPA attenuated formation of soluble Aβ dimers and oligomers after 24 h incubation (Fig. 4).

Discussion

Taken together, our data indicate that nucleation-driven aggregation of Aβ is dependent upon metal ions present at trace concentrations. Also, the coordination of metal ions to Aβ via three histidine residues in Aβ, as shown previously [44], may be the initial driving force for Aβ precipitation. The identity of the responsible metal ion(s) will require further analysis, but our data implicate Fe(III) as the contaminating metal ion most likely to initiate seeding of Aβ in the in vitro system that replicates the original report of Jarrett et al. [11], as trace concentrations of Cu(II) and Zn(II) are markedly lower than Fe(III) in the incubation buffer. We conclude that Cu(II) and Zn(II) may play only minor roles as the contaminating metal ions that initiate seeding. The possibility that other Aβ-aggregating metal ions with high affinity for DTPA, that were not assayed [e.g. Co(II) and Ni(II)], may also make a minor contribution to these reactions cannot be excluded.

Moreover, our data also show that the extended carboxyl terminus of Aβ1–42 is not solely responsible for the nucleation-driven amyloidosis of Aβ1–40, and that a metal cofactor is essential for the reaction. Interestingly, a more recent study by Sengupta et al. [45] indicates that coprecipitant(s) may be needed for in vivo Aβ aggregation since Aβ1–40 peptide is thermodynamically soluble at physiological concentrations. Their data also imply that metal ions such as Zn(II) may lower the kinetic barrier for Aβ precipitation, although they have little effects on thermodynamic solubility of Aβ peptides. Thus, our data implicate an abnormal interaction of Aβ with metal ions in AD amyloidosis if Aβ nucleation plays a role in this pathogenic event. We have focused on the amyloidosis-promoting effects of Cu(II), Zn(II), and Fe(III) in this in vitro system for three main reasons:

1. These metal ions are normally enriched in neocortical tissue most liable to amyloid deposition in AD (Cu $\sim$70 μM, Zn $\sim$350 μM, Fe $\sim$350 μM) [18], whereas other metal ions that aggregate Aβ in vitro, like Co(II) and Ni(II) [17], are either not present in biological systems in an ionic form [e.g. Co(II)] or are present at trace levels [e.g. Ni(II)] compared to Cu, Zn, and Fe.
2. Levels of Cu, Zn, and Fe are elevated in the neocortex in AD (Cu $\sim$300 μM, Zn $\sim$800 μM, Fe $\sim$700 μM), and even further enriched in amyloid plaque cores (Cu $\sim$500 μM, Zn $\sim$1300 μM, Fe $\sim$950 μM) [18, 20].
3. Levels of binding and transport proteins for Cu (e.g. ceruloplasmin) [46], Zn (e.g. alpha-2-macroglobulin) [47], and Fe (e.g. ferritin and transferrin) [48, 49] are markedly altered in AD brain.

Additionally, attenuation of metal-mediated SDS-resistant Aβ oligomerization by DTPA implicates that redox-active metals such as Cu and Fe are responsible for at least some forms of Aβ oligomerization in cell culture or even in vivo.

Aβ amyloidosis and oligomerization may be two concurrent physiochemical processes that are intrinsic to AD pathogenesis in which metals are key players. Specific metal-complexing agents, such as clioquinol (CQ), may have merit as possible therapeutics for AD treatment. Studies with CQ in APP transgenic mice have demonstrated a marked reduction of cerebral Aβ amyloid deposition [50]. Inhibition of cognitive deterioration was reported for an open label study of 30 AD patients following a 3-week treatment regime with CQ [51]. A recent pilot phase II trial of CQ with double-blind placebo control demonstrated an arrest of cognitive decline and relative decrease in plasma Aβ1–42 levels in AD subjects [52].

Ionic Cu, Zn, and Fe are exchangeably complexed to proteins and other ligands in tissue, and their levels are governed by energy- and pH-dependent homeostatic processes that may be dysregulated in AD. Our data may imply that only small percentages of the total Cu, Zn, and Fe present in AD neocortex need to inappropriately interact with Aβ in vivo and thus contribute to the nucleated growth of amyloid and Aβ oligomerization.

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